REMARKS UNDER 37 CFR § 1.111

Formal Matters

Claims 1-19 are pending after entry of the amendments set forth herein.

Claims 1-19 were examined. Claims 1-4 and 9-12 were rejected.

Claims 1 and 2 have been amended. Support for these amendments is found in the claims as originally filed, as well as in the specification at, for example: page 3, paragraph 11; page 12, paragraph 62; page 20, paragraph 20; and page 33, paragraph 132.

Claims 5 and 8 have been amended to correct a typographical error.

New claims 20-23 have been added. Support for new claims 20-23 is found in the claims as originally filed, as well as in the specification at, for example: original filed claim 8; page 4, paragraph 13 through page 5, paragraph 16; page 14, paragraph 75; and page 17, paragraph 84.

Applicants respectfully request reconsideration of the application in view of the amendments and remarks made herein.

No new matter has been added.

Examiner Interview

The undersigned Applicants' representative thanks Examiners Li and Eyler for the courtesy of an in-person interview which took place on December 16, 2003, and which was attended by Examiners Li and Eyler, Applicant Dr. Brian Kobilka, and Applicants' representatives Carol L. Francis and Edward J. Baba.

During the interview, the rejections of claims 1-4 and 9-12 under 35 U.S.C. §112 and §102, were discussed. The amendments to the claims, as well as the arguments presented herein, reflect the discussions which took place during the interview.

Restriction Requirement

Applicants acknowledge that claims 13-19 are withdrawn from consideration as being drawn to non-elected invention.

Drawings

The drawings filed on 08/21/2001 have been objected to. Replacement figures have been submitted herewith, which replacement figures comply with the requirements for formal drawings. Withdrawal of these objections is respectfully requested.

Rejection under 35 U.S.C. §112, first paragraph

Claims 1-4 and 9-12 have been rejected under 35 U.S.C. §112, 1st ¶, on the grounds that the specification failed to enable the claimed invention. In view of the remarks put forth below, this rejection is respectfully traversed.

The law is clear that "[t]he test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." United States v. Telectronics, Inc., 8 USPQ 2d 1217, 1233 (Fed. Cir. 1988), cert. denied, 490 U.S. 1046 (1989). See also, Genentech, Inc. v. Novo Nordisk, 42 USPQ 2d 1001 (Fed. Cir. 1997), cert. denied, 522 U.S. 963 (1997); Scripps Clinic and Research Foundation v. Genentech, Inc., 18 USPQ 2d 1001 (Fed. Cir. 1991).

The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. In re Certain Limited-Charge Cell Culture Microcarriers, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), aff'd sub nom., Massachusetts Institute of Technology v. A.B. Fortia, 227 USPQ 428 (Fed. Cir. 1985). See also, MPEP §2164.01. Practitioners in the chemical and molecular biology arts frequently engage in extensive modification of reaction conditions and complex and lengthy experimentation where many factors must be varied to succeed in performing an experiment or in producing a desired result. The Federal Circuit has found that such extensive experimentation is not undue in the molecular biology arts. For example, in Hybritech v. Monoclonal Antibodies, Inc. (231 USPQ 81 (Fed. Cir. 1986)) the court concluded that extensive screening experiments, while being voluminous, were not undue in view of the art which routinely performs such long experiments.

The Office Action states that the specification is enabling for a method for identifying an agonist of a GPCR, β2-adrenergic receptor (β2AR), comprising detection of a **decrease** in the fluorescent intensity of FM bound to Cys265. As Applicants understand it, the rejection is based on the assertion that the specification does not provide enablement for:

(A) detecting a **change** in a detectable signal (e.g., Office Action, page 4, first full paragraph);

(B) a method of identifying an agonist of **any** GPCR other then β2AR (e.g., Office Action, paragraph bridging pages 4 and 5),

(C) using **any** detectable signal other then a fluorescent label (e.g., Office Action, paragraph bridging pages 4 and 5), and

(D) using a GPCR having a detectable probe positioned at a residue other than one corresponding to the Cys265 residue of β-2 adrenergic receptor (Office Action, page 5 second paragraph).

These aspects of the rejection are addressed below.

(A) Detecting a change in a detectable signal

The Office Action emphasizes that the specification only provides enablement for a method of identifying an agonist of β2AR comprising detection of a decrease in the fluorescence intensity of FM bound to Cys265. The Office Action further notes that since the working examples are limited to a showing that the interaction of β2AR with an agonist results in a decrease in the detectable signal, the term "change" as used in the claims, which refers to both an increase as well as a decrease, is not enabled by the specification.

However, compliance with the enablement requirement under Title 35 U.S.C. §112, first paragraph does not require or mandate that a specific example be disclosed. The specification need not contain a working example if the invention is otherwise disclosed in such a manner that

one skilled in the art would be able to practice the invention without undue experimentation.[1] Furthermore, "[n]othing more than objective enablement is required, and therefore it is irrelevant whether [a] teaching is provided through broad terminology or illustrative examples."[2]

In the spirit of expediting prosecution and without conceding as to the correctness of this rejection, claim 1 has been amended for clarity on this point to recite "A method for identifying **a ligand** for a G protein-coupled receptor." In addition, claim 1 has also been amended to recite "wherein detection of a change in the detectable signal in the presence of the candidate agent **as compared to the absence of the candidate agent"** in order to provide a reference point for comparing the change in the detectable signal.

The specification discloses that various exemplary detectable labels including "radioisotopes, fluorophores, chemiluminescers, nitroxide spin labels or other label that provides a change in detectable signal upon a change in conformation of the GPCR" may be used. Depending on the nature of the detectable probe and the corresponding detectable signal used in the claimed invention, the interaction of the GPCR with a ligand (e.g., an agonist or an antagonist) may result in either an increase or a decrease in the detectable signal. Such will be readily appreciated by one skilled in the art upon examination of the specification of the present application.

In further support of these arguments, Applicants refer the Office to the declaration of Dr. Brian Kobilka under 37 C.F.R. §1.132. In the declaration, Dr. Kobilka provided results of experiments conducted by him or under his direction in his lab showing that the assays of the present invention can be conducted by using different detectable probes (declaration page 6, paragraph 19). For example, in Figures 1B and 1C, Dr. Kobilka provided results from experiments in which the assays of the present invention were performed using GPCRs labeled with either eosin or fluorscein and showed detection of a change in detectable signal upon ligand

1. *In re Borkowski,* 164 USPQ 640 at 645.
2. *In re Robins* 166 USPQ 552 at 555 (CCPA 1970).

interaction wherein the change can be either positive or negative, depending on the fluorophore used in the assay (see declaration at, e.g., page 7, paragraph 21).

Accordingly, the Applicants submit that the rejection of claims 1-4 and 9-12 under 35 U.S.C. §112, first paragraph, has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

(B) Identifying an agonist of any GPCR

The Office Action states that the specification does not enable the use of any GPCR other then β2AR.

It is well established that GPCRs have a conserved structure that includes seven transmembrane domains, a binding site located near the extracellular face of the receptor, and cytomplasmic domain that may be responsible for a conformational change during ligand binding. As noted in the in the Office Action, the β-2 adrenergic receptor has been described in a working example in the specification. Applicants submit that it was well known in the art at the time of filing of the present application that other GPCRs share a very similar structure to β-2 adrenergic receptor, and interact with their respective ligands and G protein signaling molecules in a similar manner. Several research publications prior to the filing of the present application establish the structurally conserved nature of GPCRs. For example, Trumpp-Kallmeyer et al., *J. Med. Chem.* (1992) 35(19):3448-62 (Exhibit 1), analyzed the structure and function of 39 different GPCRs and found strong homologies and conserved structures between the different GPCRs.

In further support of these arguments, Applicants refer the Office to the declaration of Dr. Brian Kobilka under 37 C.F.R. §1.132. In the declaration, Dr. Kobilka is asked the following questions:

> a) Is it accepted in the field that GPCRs possess a highly conserved structure and mechanism of action?
>
> b) If the answer to a) is yes, then in the context of the claimed method of detecting ligands of a GPCR, is a showing that the claimed method works the β-2 adrenergic receptor sufficient reasonably predict that the claimed method would work with other GPCRs and allow one to practice the claimed invention with other GPCRs without undue experimentation?

(declaration, page 2, paragraph 6).

In response, Dr. Kobilka states that despite the large number of genes that encode GPCRs, all GPCRs are still characterized by a highly conserved structure consisting of seven membrane spanning alpha helices with an extracellular amino terminus and an intracellular carboxyl terminus (declaration pages 2-3, paragraph 7). In addition, Dr. Kobilka notes that in spite of the diversity of ligands and ligand binding domains in the family of GPCRs, there is considerable evidence for a common mechanism of activation (declaration page 3, paragraph 8). Dr. Kobilka notes that when comparing sequences, GPCRs are most similar at the cytoplasmic ends of the transmembrane segments adjacent to the second and third cytoplasmic domains, the regions known to interact with cytoplasmic G proteins (declaration page 3, paragraph 8).

Dr. Kobilka also notes that the fact that GPCRs possess an overall structural homology at the cytoplasmic end of the transmembrane segments and the fact that all GPCRs activate highly homologous G proteins provide evidence that GPCRs undergo similar structural changes upon activation by a ligand (declaration page 5, paragraph 12). Dr. Kobilka concludes that based on such structural and mechanical similarities, it is reasonable that one of skill in the art can conclude that it will be possible to detect activation of GPCRs using a conformationally sensitive

probe positioned in the third intracellular domain, as in the method of the claimed invention (declaration page 5, paragraph 12).

Applicants further note that the methods of the present claims require that the GPCR have a conformationally sensitive third intracellular domain. Accordingly, the present claims are directed to use of only to those GPCRs that have such a conformationally sensitive third intracellular domain. Furthermore, all GPCRs function through a small set of highly homologous G proteins, with many GPCRs activating the same G protein. This further supports applicant's position that GPCRs are thus highly conserved in structure and mechanism of action. In view of the conserved structure of GPCRs, it is reasonable to conclude that once the β-2-adrenergic receptor has been shown to be susceptible to use in the claimed methods, the ordinarily skilled artisan would reasonably expect that other GPCRs having a conformationally sensitive third intracellular loop would exhibit conformational changes in response to interaction with ligand and thus can also be adapted for use in the claimed methods as set out in the specification.

To aid in determinations of enablement, courts have identified eight factors for consideration: (a) the quantity of experimentation necessary; (b) the amount of direction or guidance presented; (c) the presence or absence of working examples; (d) the nature of the invention; (e) the state of the prior art; (f) the relative skill of those in the art; (g) the predictability or unpredictability of the art; and (h) the breadth of the claims. In re Wands, 8 U.S.P.Q.2d (BNA) 1400, 1404 (Fed. Cir. 1988).

The presence or absence of working examples is but one factor to be taken into consideration in determining whether the specification is enabling for the full scope of the claims. Under MPEP § 2164.02 the consideration is whether one skilled in the art would be expected to be able to extrapolate the provided examples across the entire scope of the claim. As presented herein, Applicants argue that since the structure and function of GPCRs is highly conserved, it would be reasonable to conclude that one skilled in the art would be able to extrapolate the working examples provided in the specification across the across the entire scope of the claims.

(C) Using any Detectable Signal

Furthermore, the Office Action states that the specification does not enable the use of any detectable signal other then a fluorescent label.

The Office Action asserts that the specification only enables the use of fluorescent labels to identify an agonist of β-2-aderenergic receptor. Applicants disagree. The specification provides that the detectable probe may be photochemical, such as a fluorescent label, biochemical, or other means (see page 5, paragraph 14).

As noted above, and in accordance with the court in *Wands*, the presence or absence of working examples is one factor in determining whether a patent application has met the test of enablement. The presence or absence of working examples must be weighed against such factors as the state of the art, the relative skill of those in the art, and the predictability or unpredictability of the art. Applicants respectfully submit that the use of detectable probes well known and within the capabilities of one skilled in the art. In addition, the use of detectable probes is not a highly unpredictable field.

In further support of these arguments, Applicants refer the Office to the declaration of Dr. Brian Kobilka under 37 C.F.R. §1.132. In the declaration, Dr. Kobilka provided results of experiments conducted by him or under his direction in his lab showing that the assays of the present invention can be conducted by using different detectable probes (declaration pages 5-7, paragraphs 14-21). For example, in Figures 1B and 1C, Dr. Kobilka provided results from experiments in which the assays of the present invention were performed using GPCRs labeled with either eosin or fluorscein and showed detection of a change in detectable signal upon ligand interaction (see declaration, e.g., page 7, paragraphs 21-22).

Accordingly, Applicants respectfully assert that the specification is enabling for the full scope of the claims.

(D) Detectably Labeling Residues Other Than A Residue Corresponding to Cys265

The Office Action further asserts that the specification is only enabling for use of β2-adrenergic receptor having a detectable probe at position corresponding to Cys265.

However, the Applicants note that the disclosure of the present application specifically provides that the GPCRs may labeled with conformationally sensitive detectable probe at residues corresponding to: "1) the third intracellular loop conserved in GPCR proteins; 2) the second intracellular loop conserved in GPCR proteins; 3) amino acids in transmembrane helix 3 (TM3); and/or 4) amino acids in transmembrane helix 6 (TM6)" (page 20, paragraph 91).

In further support, Applicants submit herewith the declaration of Dr. Brian Kobilka under 37 C.F.R. §1.132. Dr. Kobilka provides results from experiments showing that β-2 adrenergic receptors in which the leucine residue at position 266 was replaced with a cysteine residue and was then labeled with a flourophore worked according to the present application, i.e. were capable of detecting a change in fluorescence properties in response to interaction with ligands (e.g., the agonist isoproterenol and the antagonist alprenolol) (declaration pages 5-7, paragraphs 14-22). Dr. Kobilka provides that, based on these experiments it is reasonable to conclude that the assays of the claimed invention can be conducted by labeling residues other than the residue corresponding to Cys265 with a detectable probe (see declaration, e.g., pages 6-7, paragraph 20).

In view of the above, the Applicants respectfully request that the rejection of claims 1-4 and 9-12 under 35 U.S.C. §112, first paragraph be withdrawn.

Rejection under 35 U.S.C. §112, second paragraph

Claims 1-4 and 9-12 have been rejected under 35 U.S.C. §112, 2nd ¶, as being indefinite for failing to particularly point out and distinctly claim the subject matter regarded as the invention. Specifically the Examiner states that claim 1 is indefinite because it recites "agonist activity" in the preamble and the steps recite "agonist binding activity," thereby allegedly

causing confusion. Claim 1 has been amended to remove the terms "binding activity." In view of the amendment to claim 1, withdrawal of this rejection is respectfully requested.

Rejection under 35 U.S.C. §102

Claims 1-4 and 9-12 have been rejected under 35 U.S.C.§102(b) as being anticipated by Gether et al., EMBO Journal 16:6737-6747, 1997 (*hereinafter* "Gether"). In view of the remarks put forth below, this rejection is respectfully traversed as applied and as it may be applied to the pending claims.

The claimed invention is directed to a method of identifying a ligand for a G protein-coupled receptor (GPCR). The method involves contacting a GPCR with a candidate agent, where the GPCR has a conformationally sensitive detectable probe positioned on or within the conformationally sensitive third intracellular domain, but not on a transmembrane domain. Ligands for the GPCR are detected by detecting a change in the detectable signal in the presence of the candidate agent as compared to the absence of the candidate agent.

In contrast, Gether merely discloses a study aimed at determining what cysteine residues of a GPCR are important in agonist induced conformational changes of the receptor. At best, Gether discloses, a series of IANBD-labeled mutant GPCRs, where certain cysteine residues were substituted to prevent their labeling by IANBD. The activity of the mutant GPCRs is compared to activity of a IANBD-labeled wild-type GPCR. Based on the results of the study, Gether concludes that agonist-induced conformational changes require the presence of transmembrane cysteines at positions 125 and 285.

Gether does not disclose or suggest a screening assay in which candidate agents may be screened to identify GPCR ligands (e.g., agonist or antagonist). In addition, it would be unreasonable to conclude from Gether's study, which was aimed at identifying cysteine residues relevant in mediating GPCR conformational changes, that one could adapt the system to screen candidate agents to identify ligands of GPCRs. Gether reported only relatively small differences between the detectable signal in the presence of a ligand compared to the detectable signal in the

absence of a ligand. Such small differences in detectable signal do not teach or suggest an assay for detecting ligands as per the claimed invention.

However, in the spirit of expediting prosecution and without conceding as to the correctness of this rejection, Claim 1 has been amended to recite "the GPCR having a conformationally sensitive detectable probe positioned on or within a conformationally sensitive third intracellular domain of the GPCR **with the proviso that the probe is not positioned in a transmembrane domain**." Support for the amendment can be found in the claims as originally filed, as well as in the specification at, for example: page 3, paragraph 11; page 12, paragraph 62; page 20, paragraph 20, and page 33, paragraph 132.

It is well established that "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." Verdegaal Bros. v. Union Oil Co. of California, 2 USPQ 2d 1051, 1053 (Fed. Cir. 1987), cert. denied, 481 U.S. 1052 (1987). See also, Scripps Clinic and Research Foundation v. Genentech, Inc., 18 USPQ 2d 1001 (Fed. Cir. 1991).

Gether does not disclose a GPCR having a conformationally sensitive detectable probe positioned on or within the conformationally sensitive third intracellular domain of the GPCR, without such a probe positioned in a transmembrane domain of the receptor. Therefore, Gether fails to anticipate the claimed invention. Furthermore, there is no suggestion in Gether that such a GPCR be made or used in a method of screening for ligands.

In view of the above, the Applicants respectfully request that the rejection of claims 1-4 and 9-12 under 35 U.S.C. §102(b) be withdrawn.

Claim Objections

Claim 1 has been objected to because of a typographical error in the claim reciting "having activity agonist activity." Claim 1 has been amended to correct the typographical error. In view of the amendment to claim 1, withdrawal of this objection is respectfully requested.

Conclusion

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number STAN-213.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: January 26, 2004

By: _____
Carol L. Francis
Registration No. 36,513

Enclosures:

1) Declaration of Brian K. Kobilka Under 37 C.F.R. §1.132
2) Supplemental Information Disclosure Statement (citing references cited in Declaration of Brian Kobilka Under 37 C.F.R. §1.132)
3) Submission of Formal Drawingss

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DECLARATION OF BRIAN K. KOBILKA UNDER 37 C.F.R. § 1.132	Attorney Docket	STAN-213
	First Named Inventor	Brian K. Kobilka
	Confirmation Number	7757
	Application Number	09/935,061
	Filing Date	August 21, 2001
	Group Art Unit	1646
	Examiner Name	Ruixiang Li
	Title: *Conformational Assays to Detect Binding to G Protein-Coupled Receptors*	

Dear Sir:

1. I, Brian K. Kobilka, declare and say I am a resident of the State of California. My residence address is 840 Chilmalus Drive, Palo Alto, California.

2. I hold an M.D. degree, which I received from Yale University in 1981. I completed my residency at Barnes Hospital, Washington University in St. Louis Missouri in 1984 and completed a research fellowship at Duke University in 1987.

3. I am a Professor of Professor of Molecular and Cellular Physiology and Medicine at the Stanford University School of Medicine. I have worked in the field of G protein coupled receptors for over 15 years and have published over 100 articles in this field. Details of my career and publications may be found in my *curriculum vitae*, provided herewith.

4. I am co-inventor of the invention claimed in the above-referenced patent application.

5. I have reviewed the Office Action dated August 26, 2003 and the rejections and objections made therein. I understand that claims 1-4 and 9-12 stand rejected under

35 U.S.C. § 112, 1st ¶, on the grounds that the specification failed to enable the claimed invention. I further understand that the rejection is based on the assertion that the specification does not provide a disclosure that would enable one of ordinary skill in the art to practice the claimed method without undue experimentation, since the only working example provided relates to the use of the GPCR β-2-adrenergic receptor having a detectable probe positioned at Cys265 (e.g., Office Action, paragraph bridging pages 4 and 5).

6. I have been asked to opine on the following questions:

a) Is it accepted in the field that GPCRs possess a highly conserved structure and mechanism of action?

b) If the answer to a) is yes, then in the context of the claimed method of detecting ligands of a GPCR, is a showing that the claimed method works with the β-2 adrenergic receptor sufficient to reasonably predict that the claimed method would work with other GPCRs and allow one to practice the claimed invention with other GPCRs without undue experimentation?

c) Can the assay of the claimed invention be conducted by labeling residues other than Cys265 with a detectable probe?

My answers to these questions is supported by the scientific literature, additional work performed in my laboratory, or both.

a) Is it accepted in the field that GPCRs possess a highly conserved structure and mechanism of action?

7. There are over 800 genes in the human genome that encode GPCRs. Despite such a large number of genes encoding GPCRs, all GPCRs are still characterized by a

highly conserved structure consisting of seven membrane spanning alpha helices with an extracellular amino terminus and an intracellular carboxyl terminus [1]. For instance, the β-2-adrenergic receptor is an example of a GPCR, which has a conformationally sensitive third intracellular domain, and yet shares the structural features conserved among all GPCRs.

8. Furthermore, in spite of the diversity of ligands and ligand binding domains in the family of GPCRs, there is considerable evidence for a common mechanism of activation. When comparing sequences, GPCRs are most similar at the cytoplasmic ends of the transmembrane segments adjacent to the second and third cytoplasmic domains, the regions known to interact with cytoplasmic G proteins [4]. Members of the large family of GPCRs transduce signals by activating one or more members of the relatively small family of highly homologous heterotrimeric G proteins. For example, the thyroid stimulating hormone (TSH) receptor is activated by a large glycoprotein hormone while the β_2 adrenoceptor is activated by adrenaline (approximately the size of a single amino acid); yet both of these receptors activate the same G protein (Gs), indicating that the structural changes in the cytoplasmic domains of these two receptors must be very similar. Moreover, many GPCRs exhibit promiscuous coupling to more than one G protein. For example, rhodopsin preferentially couples to transducin while the β-2-adrenergic receptor (β_2AR) preferentially couples to Gs; however, both are capable of activating Gi [5]. β_2AR labeled with fluorescent probes at the cytoplasmic end of TM6 (within the third intracellular domain) provide evidence that agonists induce a rotation or tilting movement of the cytoplasmic end of TM6 similar to that observed in rhodopsin [6]. Cysteine crosslinking studies have also provided evidence for a similar type of agonist-induced structural change in the M3 muscarinic receptor [7].

9. In addition, although rhodopsin is a highly specialized GPCR that is activated by light there is still evidence that the structural changes that occur in rhodopsin upon activation by light are similar to structural changes observed in other GPCRs.

Rhodopsin was the first GPCR to have been crystallized. The most recent 3D structure of rhodopsin has a resolution of 2.6Å [2]. Although high-resolution structures for other GPCRs have not yet been obtained, there is evidence that other GPCRs are structurally very similar to rhodopsin. Specifically, Ballesteros and Javitch found that structural insights obtained from mutagenesis data and substituted cysteine accessibility studies on monoamine receptors were consistent with the high resolution structure of rhodopsin, suggesting that rhodopsin serves as a good template for molecular model building for at least the largest subfamily of GPCRs [3].

10. The application asserts that GPCRs share a highly conserved structure (specification page 20, paragraph 91). These assertions are supported by literature available at the time of filing of the present application. Further research published subsequent to the filing of the present application only bolsters these assertions. Accordingly, it is accepted in the field that GPCRs possess a highly conserved structure and mechanism of action.

b) If the answer to a) is yes, then in the context of the claimed method of detecting ligands of a GPCR, is a showing that the claimed method works with the β-2 adrenergic receptor sufficient to reasonably predict that the claimed method would work with other GPCRs and allow one to practice the claimed invention with other GPCRs without undue experimentation?

11. Based on the conserved structure and mechanism of function of GPCRs and a showing that a β-2-adrenergic receptor having a detectable probe positioned on its conformationally sensitive third intracellular domain can be used in an assay to detect ligands of the GPCR, it is reasonable for one skilled in the art to predict that other GPCRs having a detectable probe positioned on their conformationally sensitive third intracellular domain can also be used in such an assays.

12. The fact that GPCRs possess a overall structural homology at the cytoplasmic end of the transmembrane segments [4] and the fact that all GPCRs activate highly homologous G proteins provides evidence that GPCRs undergo similar structural changes upon activation by a ligand. Therefore, based on such structural and mechanical similarities, it is reasonable that one of skill in the art can conclude that it will be possible to detect activation of GPCRs using a conformationally sensitive probe positioned in the third intracellular domain, as in the method of the claimed invention.

c) Can the assay of the claimed invention be conducted by labeling residues other than Cys265 with a detectable probe?

13. The following experiments were conducted by me or under my direction in my laboratory.

14. Modified β_2ARs in which five of the endogenous cysteines (Cys77, Cys265, Cys327, Cys378, and Cys406) were replaced by either serine or valine residues were then further modified to generate:

a. the mutant β2AR L266C, which has a cysteine residue at position 266 within the third intracellular domain in lieu of the native lysine;
b. the mutant β2AR S137C, which has a cysteine residue at position 137 in lieu of the native serine; and
c. the mutant β2AR A226, which has a cysteine residue at position 226 in lieu of the native alanine.

15. The modified receptors were determined to be functionally indistinguishable from the wild-type receptor.

16. The mutant β_2AR (labeled as β_2AR-S137C, β_2AR-A226C, and β_2AR-L266C) were labeled with a cysteine reactive detectable fluorophore. Since the endogenous

cysteines at positions 184, 190, and 191 are involved in disulfide bonding, and the endogenous cysteine at position 341 is palmitoylated, these residues were not available for labeling by the fluorophore. In addition, since the endogenous cysteines at positions 116, 125 and 285 are located in the transmemebrane domain the residues were not reactive towards polar fluorophores and thus were not labeled by the fluorophores. Furthermore, since the remaining five endogenous cysteines (Cys77, Cys265, Cys327, Cys378, and Cys406) had been previously replaced by either serine or valine residues, they were also not available for labeling by the cysteine reactive detectable fluorophore.

17. Therefore, only the introduced cysteine residue in each of the mutant receptors was labeled. That is, only the cysteine at residue 137 was labeled in β_2AR-S137C; only the cysteine at residue 226 was labeled in β_2AR-A226C, and only the cysteine at residue 266 was labeled in β_2AR-L266C.

18. The rate of labeling of the cysteine residues was examined under conditions of excess receptor. In addition, limited proteolysis studies were performed to confirm the specificity of the fluorophore labeling.

19. Following the labeling of each site with a panel of cysteine-reactive fluorophores, the change in fluorescence properties in response to the agonist isoproterenol and the antagonist alprenolol was tested.

20. Figures 1B-F show that all of these sites were capable of detecting ligand interaction (agonist or antagonist) through detection of structural changes in the GPCR. In particular, labeling of a cysteine residue introduced at position at 266, which is within the third intracellular domain, provided for detection of GPCR interaction with ligands. Therefore, based on these experiments it is reasonable to conclude that the assays of the claimed invention can be conducted by labeling residues other than Cys265 with a detectable probe.

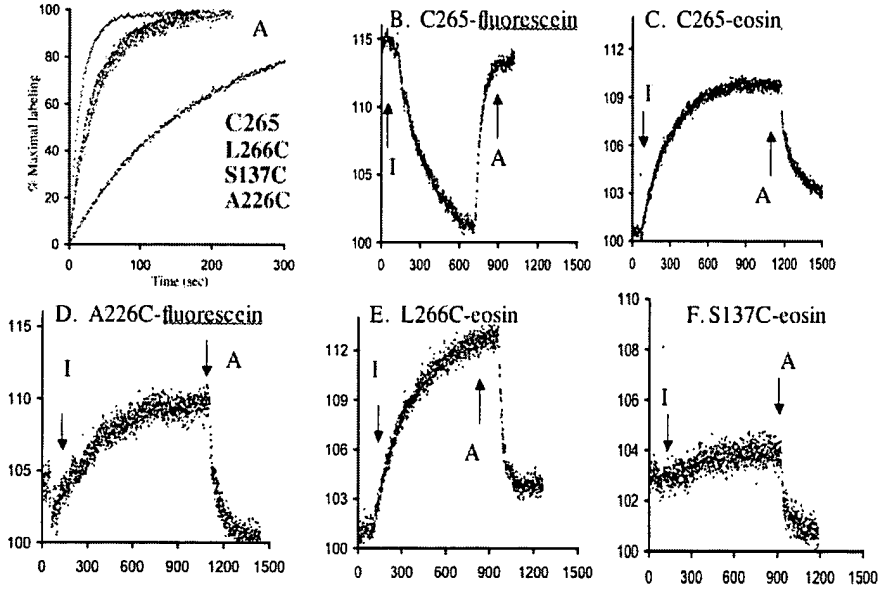

Figure 1. A. Labeling rates of indicated cysteines.
B-F. Time scans. Fluorescence response of labeled receptor to the agonist Isoproterenol (I) and the antagonist alprenolol (A).

21. Furthermore, Figures 1B and 1C show that the change in detectable signal upon ligand interaction with the GPCR can be either positive or negative, depending on the fluorophore used in the assay. For example, use of fluorescein to label residue 265 provided for a decrease in detectable signal in the presence of agonist and an incrase of signal in the presence of the antagonist, while labeling the same residue with eosin resulted in an opposite pattern of detectable change in signal.

22. Therefore, the experiments show that the assays of the present invention can also be conducted by using different detectable probes. Furthermore, the change in detectable signal can be an increase or decrease in response to ligand, depending upon, for example, the detectable label used.

23. I, Brian K. Kobilka, hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1/22/04 Brian K Kobilka

Date Brian K. Kobilka, M.D.

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